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14. ABSTRACT: The developing lymphocytes, which are a type of white blood cells, routinely create chromosome breaks while generating unique receptors to recognize foreign pathogens. The enzyme which cuts lymphocyte DNA to facilitate the construction of an immune cell receptor is encoded by two genes collectively known as the recombinase activating genes (*rag*)s. Expression of the *rag* genes is tightly controlled by cellular signals that ensure *rag* is only active in lymphocytes when immune receptor formation is occurring, after which RAG expression is shut down. Our research has revealed that mTOR controls *rag* expression in B cells by participating in a multi-protein complex called mTOR complex 2 (mTORC2). mTORC2 actively inhibits expression of the *rag* genes in B cells thereby preventing inappropriate *rag* expression and protecting the B cell DNA from excessive damage caused by *rag* activity. We found that mTORC2 suppresses *rag* expression by controlling the activity of a signaling mediator called Akt. Abnormal Akt activity is commonly associated with a wide range of cancers and our research has revealed that mTORC2 plays a key role in controlling Akt activity in B cells raising the possibility that mTORC2 inhibition may be good target for the treatment of certain B cell tumors. We show that inhibition of mTORC2 plus the chaperon protein HSP90 *in vitro* and *in vivo* elicits a potent anti-leukemic effect which is greater than inhibiting mTORC2 or HSP90 alone, suggesting that combination of mTOR inhibitors and chaperon inhibition may enhance anti-leukemic activity in blood cancer patients. Over the past year, we have explored the role of Sin1 in B cell growth and proliferation. These studies identified the pro-growth transcriptional regulator c-Myc as a target of mTORC2 mediated signaling in B cells thereby revealing a novel role for Sin1/mTORC2 in B cell growth and metabolic regulation. Finally, we utilized our unique *Sin1*$^{-/-}$ BCR-Abl leukemia cell lines to study the role of Sin1 maintaining leukemia cell viability under conditions of cellular stress such as nutrient depravation. These studies reveal that Sin1 and possibly mTORC2 play an important role in regulating the adaptive leukemic cellular response to amino acid depravation and raise the possibility that mTORC2 inhibition may sensitize leukemia cells to therapeutics that target cell metabolism.

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INTRODUCTION:

Mechanistic target of rapamycin (mTOR) is an important mediator of phosphoinositol-3-kinase (PI3K) signaling [1]. PI3K signaling regulates B cell development, homeostasis and immune responses [2]. However, the molecular mechanism of mTOR signaling in B cell growth and differentiation is not fully understood. Sin1 is a highly conserved adaptor molecule required for mTOR activation. Previous studies have established Sin1 as an indispensable component and regulator of mTOR complex 2 (mTORC2) for its integrity and function [3, 4]. We are the first to demonstrate that Sin1 is essential for mTORC2 dependent regulation of Akt and FoxO activity using mouse genetics and biochemical approaches [3]. Most importantly, our studies show that Sin1 is crucial for the integrity of mTORC2 but not mTORC1. We hypothesize that Sin1 is likely to be important for B cell tumor growth through its regulation of mTOR function. Based on our recent studies on B cell development, we also propose that Sin1 promotes genomic stability and suppresses tumor formation in developing B cell by negatively regulating recombinase activating gene (RAG) expression and Immunoglobulin (Ig) gene somatic recombination [V(D)J recombination]. As we pursued the studies outlined in this proposal we provided the first evidence linking mTOR function to the regulation of *Rag* gene expression and V(D)J recombination and developed unique and innovative new Sin1/mTORC2 deficient progenitor B cell lines and B cell leukemia cell models. Furthermore, we have explored the role of Sin1 in B cell growth and proliferation and found that Sin1/mTORC2 plays an essential role in regulating B cell growth and proliferation. These studies identified the pro-growth transcriptional regulator c-Myc as a target of mTORC2 mediated signaling in B cells thereby revealing a novel role for Sin1/mTORC2 in B cell growth and metabolic regulation. Finally, we utilized our unique *Sin1*$^{-/-}$ BCR-Abl leukemia cell lines to study the role of Sin1 maintaining leukemia cell viability under conditions of cellular stress such as nutrient depravation. These studies reveal that Sin1 and possibly mTORC2 play an important role in regulating the adaptive leukemic cellular response to amino acid depravation and raise the possibility that mTORC2 inhibition may sensitize leukemia cells to therapeutics that target cell metabolism.

BODY:

We have largely completed the key goals outlined in the SOW of this proposal and, in the process of completing these goals, revealed new and unexpected functions of Sin1 in B cell growth, development and immune function. Briefly, we completed the primary studies outlined in **Task 1** in year 1 of the proposal. In **Task 1**, we hypothesized that Sin1 regulates RAG expression and Ig gene V(D)J recombination through a mechanism involving mTORC2. We found that Sin1 is required for B cell development and Ig gene recombination and showed that Sin1, as part of mTORC2, suppresses Ig gene V(D)J recombination by regulating the expression of the RAG recombinase genes *rag1* and *rag2*. Mammalian TORC2 mediates pre-B cell receptor or B cell receptor signaling and phosphorylates Akt2 at S473. S473 phosphorylated Akt2 phosphorylation directs Akt2 substrate specificity towards the transcriptional regulator FoxO1 resulting in the inactivation of FoxO1 and reduction of FoxO1 dependent RAG gene expression. The increased RAG expression in Sin1 deficient B cells correlated with increased V(D)J recombinase activity as measured by an artificial virally expressed V(D)J recombinase substrate and by increased Ig light chain receptor editing in Sin1 deficient immature B cells.

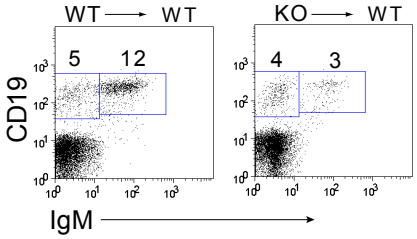

Figure 1: Bone marrow from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ chimeric mice was analyzed by flow cytometry and the percentage of CD19^{+} IgM^{-} and CD19^{+} IgM^{+} B cells is indicated. The data are representative of *Sin1*$^{+/+}$ (n=3) and *Sin1*$^{-/-}$ (n=4) fetal liver chimeric mice.

In year 3 we completed the goals outlined in **Task 2**. In this **Task**, we set out to elucidate the function of Sin1 in B cell growth and development. To achieve our goals we successfully developed an *in vivo* model to study Sin1 function in the immune system of mice by adoptively transferring *Sin1*$^{-/-}$ fetal liver hematopoietic stem cells into lethally irradiated wild type or *rag1*$^{-/-}$ recipient mice (**Task 2b**). Additionally, we successfully developed an *in vitro* pro-B cell culture system, as outlined in **Task 2c**, to facilitate the study of Sin1 function in pro-B cells and in bone marrow B cell differentiation. Both of these model systems were developed by first isolating *Sin1*$^{-/-}$ or *Sin1*$^{+/+}$ fetal liver hematopoietic cells

(FL-HSCs) from day 11.5-12.5 mouse embryos. Our chimeric mouse model was created by adoptively transferring FL-HSCs into lethally irradiated wild type CD45.1^{+} congenic mice or *rag1*$^{-/-}$ mice. In the CD45.1^{+} chimeras, lymphocytes arising from the FL-HSCs are easily distinguished from the host lymphocytes by flow cytometry using the differential expression of the cell surface markers CD45.1 (exclusively expressed on host cells) and CD45.2 (expressed on donor cells). While in the *rag1*$^{-/-}$ chimeras, all lymphocytes present in these animals arose exclusively from the FL-HSCs since the RAG1 mutation completely blocks host lymphocyte development. *Sin1*$^{-/-}$ FL-HSCs successfully engrafted and contributed to the long term reconstitution of the hematopoietic system of both strains of recipient mice. Analysis of the bone marrow and spleen of *Sin1*$^{-/-}$ FL-HSC chimeric mice revealed that loss of Sin1 is required for B cell development. Sin1 deficiency results in a reduction of IgM^{+} immature B cells in the bone marrow (**Fig. 1**) and impaired development of mature peripheral B cells with a specific loss of the splenic marginal zone B cells and the loss of peritoneal B1a B cells (**Fig. 2**). These data clearly show that Sin1 is required for B cell development.

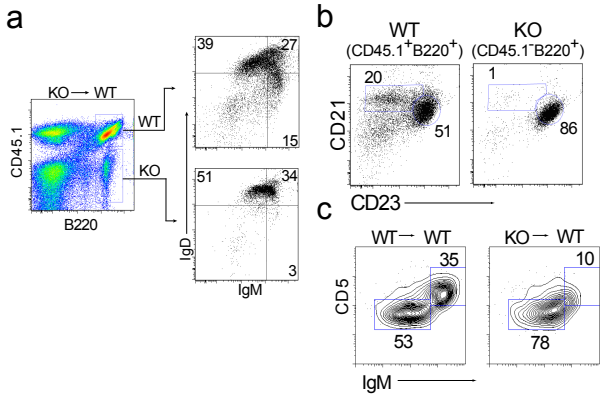

Figure 2: **a)** Total splenocytes from a *Sin1*$^{-/-}$ fetal liver HSC chimeric mouse were stained with the indicated antibodies and analyzed by flow cytometry. Host wild type (WT) B cells (CD45.1^{+}B220^{+}) and donor *Sin1*$^{-/-}$ (KO) B cells (CD45.1^{-}B220^{+}) were gated and analyzed for IgM and IgD expression. The proportion of host and donor IgM^{hi}IgD^{low}, IgM^{hi}IgD^{hi} and IgM^{low}IgD^{hi} splenic B cells is indicated next to each respective quadrant. The data shown are representative of three individual *Sin1*$^{-/-}$ fetal liver HSC chimeric mice. **b)** The proportion of WT and KO marginal zone B cells (CD21^{high}CD23^{low}; rectangular gate) and follicular B cells (CD21^{int}CD23^{hi}; circular gate) in the spleen of the *Sin1*$^{-/-}$ chimeric mouse described in **a** are indicated next to the corresponding FACS gates. The plots shown are pre-gated on B220^{+}CD45.1^{+} host wild type (WT) or B220^{+}CD45.1^{-} donor *Sin1*$^{-/-}$ (KO) B cells and are representative of three *Sin1*$^{-/-}$ fetal liver HSC chimeric mice. **c)** FACS plots showing donor derived B cells obtained from the peritoneal cavity of a *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ fetal liver HSC chimeric mice were stained for CD5 and IgM. The plots shown are pre-gated on CD45.1^{-} CD19^{+} donor B cells and are representative of n=2 *Sin1*$^{+/+}$ and n=2 *Sin1*$^{-/-}$ chimeric mice. The percentage of B1a B cells (CD5^{high}IgM^{high}) were reduced in Sin1 deficient chimeric mice when compared to WT chimeric mice (10% vs. 35% respectively).

Sin1 plays an essential role in the mTOR signaling pathway. We hypothesized that the loss of Sin1 may impair mTOR signaling in B cells and result in defects in B cell growth and proliferation. Our analysis of peripheral B cells obtained from the *Sin1*$^{-/-}$ FL-HSC chimeric mice revealed that mature splenic and lymph node B cells lacking Sin1 were indeed smaller than wild type B cells (**Fig. 3**). These data indicate that Sin1 may play an important role in regulating B cell metabolism and/or macromolecular synthesis. Cell growth is essential for the development of a functional B cell immune response. Therefore, we asked if Sin1 is also required for the blast cell growth and proliferation of mitogen activated B cells. We stimulated splenic B cells isolated from *Sin1*$^{-/-}$ or *Sin1*$^{+/+}$ FL-HSC chimeric mice with lipopolysaccharide (LPS), a potent polyclonal B cell mitogen, and measured B cell growth and proliferation. We found that Sin1 deficient B cells showed severely impaired blast cell growth and proliferation in response to LPS stimulation (**Fig. 4**). We observed similar results in *Sin1*$^{-/-}$ B cells stimulated thorough the B cell receptor which strongly suggests that Sin1 plays an important role in mediating mitogen dependent signals that promote B cell growth and immune function. Surprisingly, we found that Sin1 is not required for the growth and proliferation of T cells indicating that Sin1 and, most likely, mTORC2 play a specific and key role in regulating B cell growth and immunity.

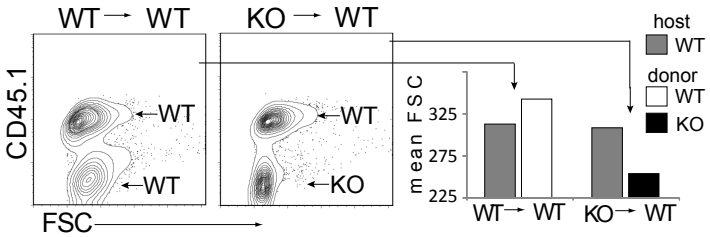

Figure 3: The relative size of splenic B cells from *Sin1*$^{+/+}$ (WT-->WT) or *Sin1*$^{-/-}$ (KO-->WT) fetal liver HSC-chimeric mice was measured by forward light scatter (FSC). The fetal liver HSC derived CD45.1^{-} WT or KO B cells (donor) and WT CD45.1^{+} (host) B cell populations within each mouse are indicated. The plots shown are pre-gated on live, CD19^{+} lymphocytes and are representative of n=2 WT and n=3 KO chimeric mice.

In order to elucidate the molecular mechanism of Sin1 function in B cell growth, we turned to our *in vitro Sin1*$^{-/-}$ pro-B cell culture system. Primary pro-B cell lines were derived from the fetal liver cells of *Sin1*$^{-/-}$

or *Sin1*$^{+/+}$ litter mate E12.5 embryos and then cultured on a confluent monolayer of OP9 mouse bone marrow stromal cells in the presence of recombinant mouse IL-7. These culture conditions give rise to a phenotypically pure population progenitor *Sin1*$^{-/-}$ or *Sin1*$^{+/+}$ B cells which can be expanded and cultured for an extended period of time. We then plated these pro-B cell lines on a new confluent OP9 monolayer without exogenous IL-7 to induce B cell differentiation. After 5-7 days, these differentiating B cells cultures give rise to populations of developing B cells that resemble bone marrow IgM$^-$ pre-B, IgM$^+$ immature B and IgM$^+$IgD$^+$ transitional B cells. We measured the cell size of pro-B, pre-B, immature B and transitional B cells from these cultures and found that Sin1 deficiency results in a decrease in B cell size at the later immature and transitional B cell developmental stages but not at the earlier pro-B and pre-B developmental stages (**Fig. 5**). These data reveal that Sin1 regulates B cell growth in a developmental stage specific manner.

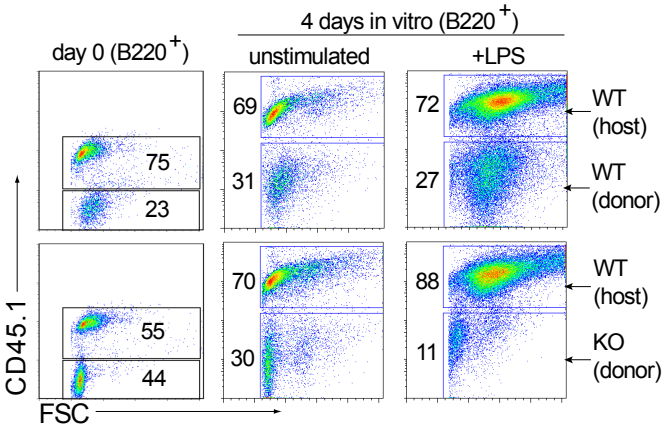

Figure 4: Total splenocytes from *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) fetal liver HSC chimeric mice were cultured in vitro with medium alone (unstimulated) or with 10 µg/ml LPS (+LPS) for 4 days. The relative size of the WT CD45.1$^+$ (host) B cells and CD45.1$^-$ (donor) WT or KO B cells was measured by FSC. The plots show freshly isolated splenic B cells (day 0) and splenic B cells cultured in vitro for 4 days with or without LPS. All of the plots shown are pre-gated on live, B220$^+$ B cells and the numbers adjacent to each gate indicate the percent of B cells within the gate.

Our discovery that Sin1 regulates the cell size of developing and mature B cells strongly suggested that Sin1, and possibly mTORC2, may act as an upstream regulator of one or more key growth or metabolic regulators in B cells. The transcription factor c-Myc is a potent enhancer of B cell growth and metabolism whose expression is normally up-regulated in proliferating B cells. In addition, the abnormal up-regulation of c-Myc expression is also a common feature of many B cell cancers. Therefore we examined c-Myc expression in *Sin1*$^{-/-}$ B cells and found that c-Myc expression was significantly lower in *Sin1*$^{-/-}$ B cells when compared to *Sin1*$^{+/+}$ B cells. We found that Sin1 was not required for c-Myc gene expression indicating that Sin1 regulates the c-Myc protein translation or degradation (**Fig. 6a & 6b**). In addition, the mTOR kinase inhibitor pp242, which inhibits both mTORC1 and mTORC2, completely blocked the up-regulation of c-Myc expression in activated B cells whereas rapamycin, which only inhibits mTORC1, partially blocked the up-regulation of c-Myc expression in B cells (**Fig. 6c & 6d**). Together, these data indicate led us to hypothesize that Sin1/mTORC2 regulates c-Myc expression through a mechanism which is independent of mTORC1. Indeed, we found that mTORC2 signaling promotes the stability of c-Myc protein through a pathway which is dependent on Akt and GSK3. These data reveal a novel role for Sin1/mTORC2 in the regulation of c-Myc dependent B cell growth.

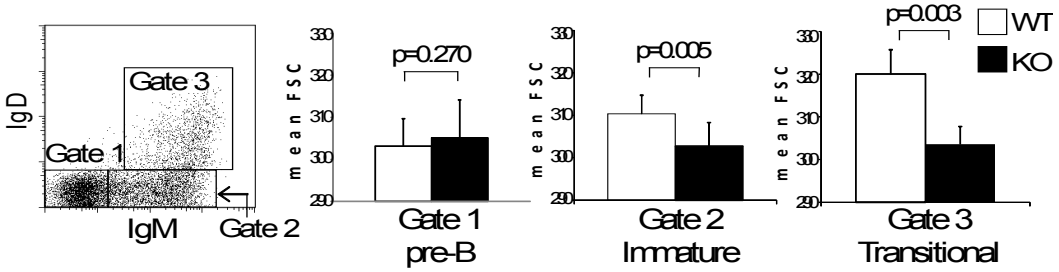

Figure 5: *Sin1*$^{+/+}$ (WT) and *Sin1*$^{-/-}$ (KO) pro-B cells were differentiated in vitro for 7 days on OP9 stromal cells and the mean FSC of pre-B (Gate 1), immature B (Gate 2) or transitional B (Gate 3) cells was measured by flow cytometry. The bar graphs for each of the gated B cell populations are the average FSC measurements from 4 independent experiments with standard deviation indicated by the error bars.

Our experiments proposed in **Task 3**, led us to explore the role of Sin1 in regulating the response of leukemic B cells to nutrient and energy depravation. Mechanistic TOR is a master regulator of cellular metabolism which may activate an adaptive starvation response when cells are deprived of nutrients (i.e. amino acids) or energy (i.e. glucose). To explore the role of mTORC2 in regulating the cellular response to nutrient or energy depravation, we derived pre-B leukemia cells from *Sin1*$^{+/+}$ or *Sin1*$^{-/-}$ pro-B cells through transformation with the human oncogene p210BCR-Abl. Interestingly, Sin1/mTORC2 is not required for BCR-Abl leukemia cell growth, proliferation or survival when nutrients and energy sources are abundant. However, we found that *Sin1*$^{-/-}$ BCR-Abl leukemia cells showed significantly reduced survival compared to *Sin1*$^{+/+}$ leukemia cells when subjected to conditions of amino acid starvation. We found that *Sin1*$^{+/+}$ p210BCR-Abl leukemia cells grown in low cysteine or low glutamine medium were capable of maintain their cell size under amino acid starvation conditions however, *Sin1*$^{-/-}$ p210BCR-Abl pre-B leukemia cells underwent a significant reduction in cell size when subjected to starvation conditions (**Fig. 7**). Consequently, *Sin1*$^{+/+}$ leukemia cells were able to continue to proliferate, albeit at a slower rate, under nutrient starvation conditions while *Sin1*$^{-/-}$ leukemia cells failed to proliferate and ultimately died under these same conditions. These studies reveal a novel role for Sin1 in the regulation of the leukemic cell response to nutrient stress. We are currently pursuing the underlying mechanism through which Sin1 mediates this cellular adaptation to nutrient depravation and will determine if Sin1 acts through mTORC2 to mediate this cellular stress response.

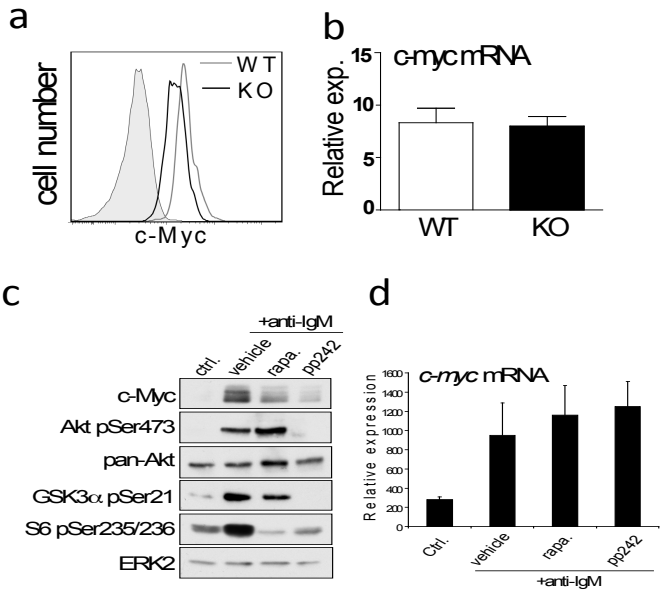

Figure 6: **a)** *Sin1*$^{+/+}$ (WT) or *Sin1*$^{-/-}$ (KO) immature B cells were stained and c-Myc expression was measured by intracellular staining and flow cytometry. The shaded line is the negative staining control. **b)** The expression level of *c-myc* mRNA was then measured by quantitative (q)RT-PCR in WT or KO IgM^{+} B cells. Each sample was normalized to the expression of GAPDH. **c)** Wild type splenic B cells were enriched by negative selection and pretreated for 15 minutes with vehicle only (vehicle), 20 nM rapamycin or 50 nM pp242, and then cultured for an additional 60 minutes with medium only (ctrl.) or 10 μg/ml anti-IgM F(ab')$_2$. The expression level and phosphorylation status of the indicated cellular proteins was measured by immunoblotting. **d)** Wild type splenic B cells were pre-treated with indicated mTOR inhibitors and then stimulated with anti-IgM F(ab')$_2$ as described in panel **a**. Relative expression levels of *c-myc* mRNA was measured by qRT-PCR and normalized to the expression level of GAPDH.

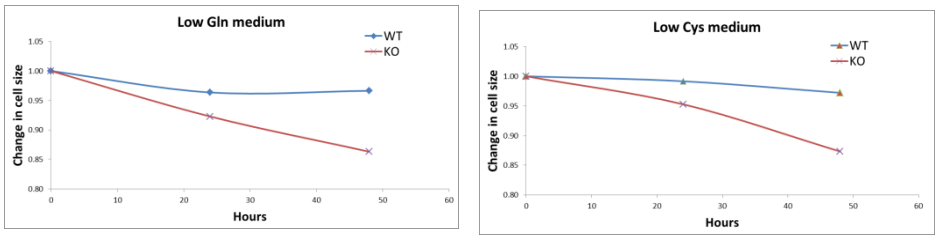

Figure 7: Sin1$^{+/+}$ and Sin1$^{-/-}$ p210BCR-Abl were co-cultured together in cell culture medium which was specifically deficient in glutamine or cystine. The change in cell size relative to 0 hrs was determinded by FSC using flow cytometry and Sin1$^{+/+}$ and Sin1$^{-/-}$ cells were distinguished by the differential expression of GPF.

KEY RESEARCH ACCOMPLISHMENTS:

- Sin1 is required for the development of peripheral mature B cells
- Sin1 regulates B cell growth in a cell type specific and cell developmental stage specific manner
- Mammalian TORC2 regulates B cell growth through c-Myc and mTORC1

- Sin1 promotes BCR-Abl B cell leukemia growth under conditions where nutrient availability is limiting

REPORTABLE OUTCOMES:

Manuscripts:

Adam S Lazorchak, Omotooke A Arojo, Xiaocao Xu, Dou Liu, Fei Tang, Pan Zheng & Bing Su. Sin1/mTORC2 coordinates c-Myc and mTORC1 anabolic activities to regulate B cell size. Manuscript submitted.

CONCLUSION:

DNA damage which results in the breaking of a chromosome is inherently dangerous to a cell and may result in mutations which are a common cause of blood cancers such as leukemia and lymphoma. The developing lymphocytes, which are a type of white blood cells, routinely create chromosome breaks while generating unique receptors to recognize foreign pathogens. The enzyme which cuts lymphocyte DNA to facilitate the construction of an immune cell receptor is encoded by two genes collectively known as the recombinase activating genes (*rag*)s. Expression of the *rag* genes is tightly controlled by cellular signals that ensure *rag* is only active in lymphocytes when immune receptor formation is occurring, after which RAG expression is shut down. Over the past year we have investigated how a specific mediator of cellular signals called mammalian target of rapamycin (mTOR) regulates *rag* expression in B lymphocytes. Our research has revealed that mTOR controls *rag* expression in B cells by participating in a multi-protein complex called mTOR complex 2 (mTORC2). mTORC2 actively inhibits expression of the *rag* genes in B cells thereby preventing inappropriate *rag* expression and protecting the B cell DNA from excessive damage caused by *rag* activity. Our studies have revealed that mTORC2 suppresses *rag* expression by controlling the activity of a signaling mediator called Akt. Abnormal Akt activity is commonly associated with a wide range of cancers and our research has revealed that mTORC2 plays a key role in controlling Akt activity in B cells raising the possibility that mTORC2 inhibition may be good target for the treatment of certain B cell tumors. Over the past year we have explored how Sin1 and mTORC2 regulates the growth of B cells. We have revealed a novel role for mTORC2 in the regulation of B cell growth and shown that the proto-oncogene c-Myc is a target of Sin1/mTORC2 regulation. We have also explored the role of Sin1 the adaptive response of leukemia cells to nutrient depravation stress. These studies have revealed a surprising new role for Sin1 as a regulator of cellular growth under conditions of nutrient stress. We are currently exploring the molecular basis of this Sin1 dependent nutrient stress response which we strongly believe will provide new insights to guide the development of novel therapeutics to treat blood cancers.

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APENDECESE:

None.